Field Trials To Determine Residues of Chlozolinate in Table Grapes

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Chlozolinate (Serinal) is a dicarboximide fungicide used in southern European countries principally on grapes. Maximum residue levels have not yet been set by FAO/WHO and are under evaluation in the EU. Field trials have been carried out in Greece on two varieties of table grapes (Cardinal and Victoria) during two consecutive years to assess residues remaining after application according to good agricultural practice. Analysis using a multiresidue method with gas chromatography (ECD) showed that the parent compound decays with a first-order rate constant of 0.057 ± 0.011 day^{-1} and that residues had fallen below the proposed MRL of 5 mg/kg in all samples by 21 days postapplication (the proposed PHI). The contribution of the main metabolite, S1, to the total residue is generally <20%. Washing removes a substantial amount (up to 80%) of chlozolinate, which appears to be nonsystemic on grapes, thus reducing real consumer exposure to this pesticide.

Keywords: *Chlozolinate; residues; grapes; washing; dissipation*

INTRODUCTION

Chlozolinate (trade name Serinal; IUPAC chemical name and structure given in Figure 1) is a fungicide of the dicarboximide group with protective and curative action. It acts through inhibition of mycelial growth and, to a lesser extent, conidial germination of sensitive fungi. It is used on stone fruits, strawberries, grapes, and ornamentals against *Monilia*, *Botrytis*, *Sclerotium*, *Sclerotinia*, *Gloesporium*, and *Colletotrichum*. In southern European countries the most important use is on table and wine grapes. Because table grapes make an important contribution to the human diet, data on the magnitude of chlozolinate residues after application according to good agricultural practice (GAP) are essential both for the establishment of tolerances (maximum residue levels, MRLs) and for the calculation of the theoretical maximum daily intake. Furthermore, data on the effect of washing on residue concentration are required to assess real consumer exposure. Chlozolinate is a relatively new compound, and literature data available on residue levels are limited to artichokes (Corde et al., 1985), tomatoes (Cabras et al., 1985), and wine grapes (Cabras et al., 1983, 1984; Gennari et al., 1992). This paper reports the results of trials with two varieties of table grapes carried out in Greece during two consecutive years.

MATERIALS AND METHODS

Chemicals. All solvents and reagents were of high purity and suitable for pesticide residue analysis. Chlozolinate formulated material (Serinal 50% WP) and analytical standards of the parent compound and three metabolites, S1, S2, and S3 (Figure 1), were kindly provided by ISAGRO.

Field Trials. The trials were carried out in 1996 and 1997 in a northern suburb of Athens, Lycovrissi, on an experimental field belonging to the National Agricultural Research Foundation (N.AG.RE.F.) The fungicide was applied on two varieties of table grapes. The vines were relatively low (reaching up to 40−50 cm above the ground): Cardinal, an early red-violet variety with loosely packed berries (22-year-old plants), and Victoria, a later yellowish-green variety with tightly growing berries (18-year-old plants). Both varieties are grafted on Richter 110 rootstock and are grown on a well-drained clay soil of pH 7.2−7.8 and organic matter content of 2.0−2.5%. Regular agricultural practice was carried out on the plots (phosphorus and potassium fertilization in December, bush pruning in February, and nitrogen fertilization in March). Chemical weed control was carried out using glyphosate, powdery mildew was controlled with Afugan (pyrazophos), and insects and mites were controlled with Gusathion (azinphos-methyl). Tipping took place early in June, and leaf removal was carried out twice, before the second and the last sprayings. The experimental plots in 1996 consisted of 16 Cardinal plants and 32 Victoria plants, whereas in 1997 they were extended to include 65 and 60 plants, respectively. The minimum number recommend by the guidelines of the Commission of the European Communities (ECa, 1997) is 4. A number of plants—16 Cardinal and 7 Victoria—were left untreated to be used as controls. In 1996, the lower recommended number of treatments (three) was carried out on June 4, July 1, and July 15 for Cardinal and on June 4, July 15, and August 1 for Victoria, dates that corresponded to the recommended growth stages, that is, the end of blooming, berry set, and veraison. The application rate was the lower recommended one of 0.075 kg of active substance (a.s.)/hL. Taking into account the volume of water used per square meter, the application rate is estimated to be 0.75 kg of a.s./ha. The application was carried out by knapsack sprayer until run off. In 1997, the higher recommended number of treatments (four) was carried out on June 12, July 1, 11, and 31 for Cardinal and on June 12 and 30, July 21, and August 4 for Victoria, corresponding to the recommended growth stages as referred to above. The application rate was the higher recommended one of 0.100 kg of a.s./hL, giving an estimated application rate of 1.00 kg of a.s./ha, which is similar to the critical GAP in the south of Europe (0.105 kg of a.s./hL, i.e., 1.050 kg of a.s./ha., four sprays). The application was carried out by a tractor-mounted single-nozzle sprayer. In both years, care was taken to ensure that both foliage and grapes were well covered with the spraying

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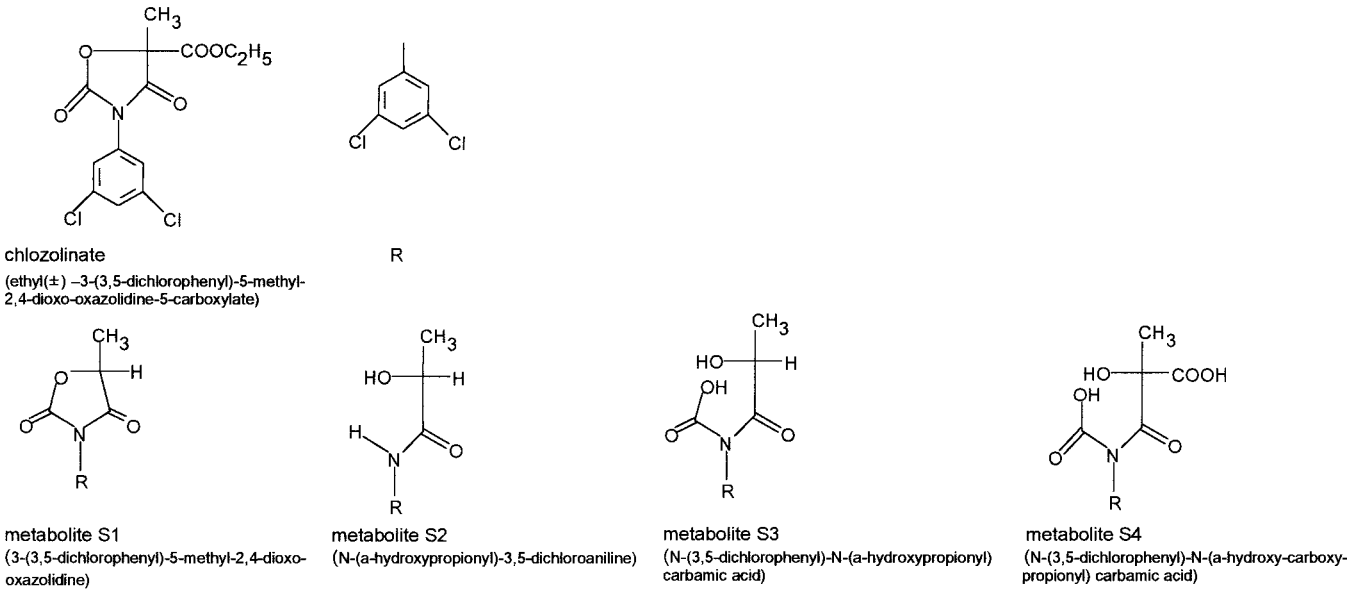

Figure 1. Chemical structures of chlozolinate and its metabolites S1, S2, S3, and S4.

Table 1. Recoveries of Chlozolinate and Metabolite S1 from Fortified Samples[a]

fortification level	chlozolinate (percentage recovery in replicates)							S1 (percentage recovery in replicates)						
(mg/kg)	1	2	3	4	5	mean	RSD	1	2	3	4	5	mean	RSD
10	84	82	83	85		84	2	53	51	55	52		53	3
5	82	88	88	87	86	86	3	48	53	57	55	59	54	8
1	96	98	98	100	99	98	2							
0.4	89	80	86	88	88	86	4							
0.1	96	93	95	95	100	96	3							
0.02								58	49	52			53	9
0.04	91	97	96	94	100	96	4							
0.004	93	95	93	92	92	93	1							
mean recovery over all fortification levels						91	6						53	7

[a] Matrix-matched standards were used for all GLC analyses.

mixture. The meteorological data for the growing periods in 1996 and 1997 are shown in Figure 2. No irrigation was applied.

Sampling. Samples were collected at time 0 (2−3 h post-application, when the spraying mixture had dried) and 4, 8, 14 or 15, 21, and 28 days after the last application. The recommended preharvest interval (PHI) is 21 days. In the first year, the samples consisted of 12−14 bunches, with 12 the minimum number recommended in the Commission of the European Communities guidelines (ECa, 1997). In the second year the number of bunches, 40−50, was much higher. After weighing, the bunches of each sample were generally cut into smaller parts (some bunches of Cardinal in 1996 had too few berries for subdivision), and the sample was divided into six subsamples. Three subsamples were then homogenized as such in a Waring blender after removal of the stems, as recommended by the Council Directive of the European Communities (EEC, 1990). The other three subsamples were subjected to washing by submerging them for 10 min in a volume of water sufficient to just cover the sample. After removal from the water, they were placed on filter paper for a few minutes to drain and then homogenized. To assess the effect of the growth of berries on the residue concentration, 100 berries from each sample were taken at random and weighed. For samples of <100 berries, all berries were weighed and the mass was adjusted proportionally.

Analysis. Chlozolinate is a compound of intermediate polarity (log $P_{o/w}$ = 3.15). In grapes it is metabolized by hydrolysis and decarboxylation to a number of metabolites, S1, S2, S3, and S4 (Figure 1), the most important being S1 (Santi et al., 1983; Tomlin, 1994). Because the contribution of the sum of these metabolites to the total residue is generally low (<10%) and their toxicity is less than that of the parent compound because of their greater polarity (ECb, 1997), the parent compound is a good indicator of the total residue. Most of the methods used in the published literature for the determination of chlozolinate residues are based on the method of Cabras et al. (1983). This is an HPLC method enabling the simultaneous determination of several dicarboximide fungicides (vinclozolin, iprodione, procymidone, and chlozolinate) and a common degradation product, 3,5-dichloroaniline. Pirisi et al. (1986) have found that the degradation of chlozolinate in wine was accompanied by the formation of the metabolite S1. Gennari et al. (1992) adapted the above HPLC method to cover this metabolite. No GLC method is reported in the literature. A GLC method developed by the manufacturer (not published) uses TSD with a limit of determination (LOD) of 0.01 mg/kg. As chlozolinate contains two chlorine atoms in its molecule, it is reasonable to assume that the use of ECD will lower the limit of determination. A study has been carried out in our laboratory on the possibility of including chlozolinate in an ECD multiresidue method based on that published by the Ministry of Welfare, Health and Cultural Affairs, The Netherlands (1988) (Lentza-Rizos and Avramides, 1999). This involves the extraction of 25 g of homogenized sample with 50 mL of toluene and 25 mL of propan-2-ol using an Ultra-Turrax, removal of propan-2-ol by washing twice with water (125 mL of 2% Na_2SO_4 solution each time), and cleanup of the pesticide-containing toluene phase with an adsorbent mixture of Celite and activated charcoal (1:3, w/w). After filtration, the residues are determined by gas chromatography with ECD. Recovery tests for chlozolinate from grapes over a wide range of fortification levels (0.004−10 mg/kg) gave a mean percentage recovery of 91 with an RSD of 6 (Table 1) and an LOD of 0.004 mg/kg. The main metabolite, S1, was also tested with this method. The average percentage recovery for three fortification

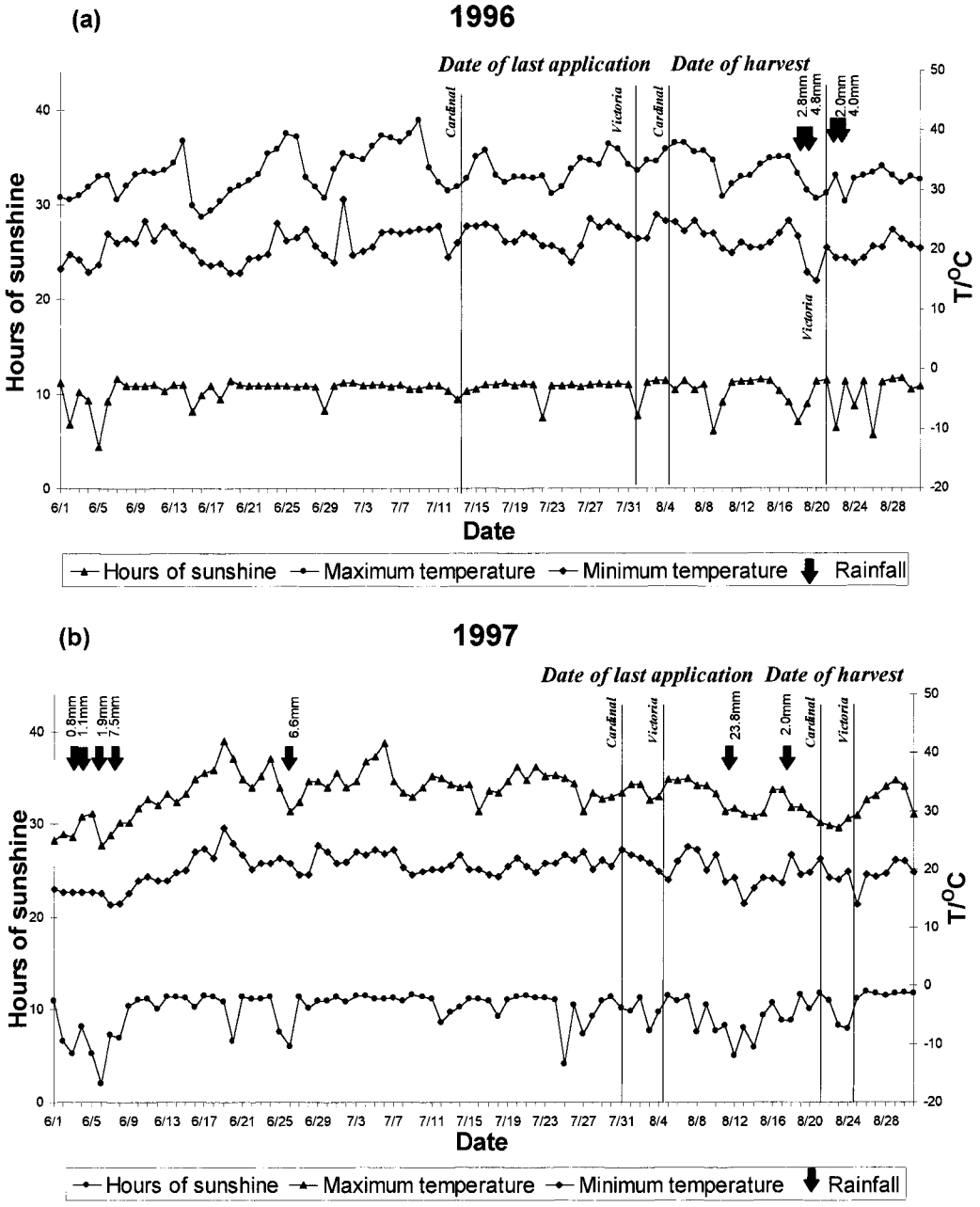

Figure 2. Meteorological data for the growing periods in (a) 1996 and (b) 1997.

levels, 0.02, 1, and 2 mg/kg, with a minimum of three replicates at each level, was 54 with an RSD of 6. All standard solutions used for GLC analysis were matrix-matched. The metabolites S2 and S3 were not recovered and are in any case difficult to determine using GLC. Although the recovery for S1 is low, the RSD is correspondingly small and the results are reported (without correction for recovery) because they give useful information on the total residue behavior. The GLC analyses were carried out using a Hewlett-Packard 5890 series II gas chromatograph with an ECD detector (300 °C), splitless mode injection (250 °C, 60 s, 1 μL), and chromatographic columns Rtx-5 (30 m, 0.25 mm i.d., 0.25 μm film thickness, 5% diphenyl, 95% dimethyl polysiloxane) and HP608 (30 m, 0.53 mm i.d., 0.5 μm film thickness). All analyses were carried out in duplicate.

RESULTS AND DISCUSSION

Initial Deposits. The concentration of the parent compound at time 0 in the three subsamples of Cardinal ranged from 3.36 to 5.81 mg/kg in 1996 (three treatments, lower application rate, knapsack sprayer) and from 10.2 to 12.3 mg/kg in 1997 (four treatments, higher application rate, tractor-mounted sprayer). The respective values for Victoria were 4.10−5.83 mg/kg in 1996 and 9.23−12.2 mg/kg in 1997. Mean concentrations are given in Table 3. The higher initial deposits in 1997 were to be expected because of the larger number of treatments, the higher application rate, the more efficient mode of application, and the smaller average size of the berries, that is, larger surface-to-mass ratio (Table 2). The metabolite S1 was detected in all samples even at time zero (2 h postapplication), suggesting that it is formed rapidly after application or that the formulated product already contained S1. It is also probable that small amounts of the metabolite were still present from previous treatments. However, data on the effect of washing on the metabolite residues (see below) indicate that part of the initial deposit of S1 had been freshly formed. The variation in chlozolinate residue concentra-

Table 2. Masses (Grams) of Samples Analyzed and of 100 Berries from Each Sample

days post-application	Cardinal				Victoria			
	sample		100 berries		sample		100 berries	
	1996	1997	1996	1997	1996	1997	1996	1997
0		6955	282	338	2897	7535	487	307
4	2543	6501	321	342	2824	6196	455	292
8	2225	5006	333	324	3151	8691	478	356
14 or 15	2133	6994	402	370	4092	8022	540	320
21	3887	5294	459	362	5102	13321	622	353
28	8544	7902	437	333	3622	7617	446	285
p (linear regression)			0.0001	0.405			0.527	0.946

tion between subsamples was higher in 1996 than in 1997 (RSD for Cardinal = 31% and RSD for Victoria = 17% compared to 9 and 15%, respectively). This is attributed, first, to the fact that in 1997 the sample size was considerably larger and, second, to the application mode, with that used in 1997 likely to give more uniform coverage of plants. Comparison of the initial deposits on the two varieties using an unpaired t test showed no significant difference between them in either 1996 or 1997.

Effect of Growth on Residue Dissipation. Regression analysis of the mass of 100 berries versus time reveals a statistically significant ($p < 0.05$) influence of growth only in the case of Cardinal in 1996 (Table 2). This observation is consistent with the fact that, in this case, the fungicide was last applied in the middle of July when berry size was still increasing, rather than 2−3 weeks later as in the other three experiments. The regression analysis line of the data from day 0 to day 21 ($r^2 = 0.95$) was used to correct the residue data for the dilution effect of growth so that data from the two years of the study could be compared. By day 21 (the beginning of August), comparison with the other sets of data appears to confirm that growth of the berries had ceased.

Dissipation with Time. Residues of chlozolinate and S1 at the different sampling times are given in Tables 3 and 4, respectively. The dissipation curves of chlozolinate (parent compound only) are shown in Figure 3. For Cardinal grapes in 1996, the curves with and without correction for growth are given. The first-order rate constants for the two years for each variety are in good agreement, giving mean half-lives of 11 days for Cardinal and 14 days for Victoria. The slightly higher rate constants found for Cardinal grapes may be associated with the loose packing of the berries of this variety, which make it more likely that climatic conditions will affect residues. The exceptionally heavy rainfall (23.8 mm) that occurred 12 days after the final treatment of Cardinal in 1997 may, therefore, account for the higher rate of dissipation observed for this year. All rate constants fall within the range $0.01 < k_{obs} < 0.1$, leading to the classification of chlozolinate as a moderately persistent compound (Hoskins, 1961). The concentration of metabolite S1 increases up to 4−8 days postapplication and then begins to decrease. It does not vary greatly with time and is generally <20%.

Residues at Proposed PHI. The mean residues (parent compound) determined on the grapes 21 days postapplication are given in Table 3. The ranges of values found for the three subsamples in 1996 and 1997, respectively, are 0.56−0.87 and 2.81−4.05 mg/kg for Cardinal and 1.45−2.00 and 3.80−4.60 mg/kg for Victoria. The concentrations determined on and in each individual sample are below the value of 5 mg/kg proposed by EU experts as the MRL on the basis of critical GAP. S1 constitutes a relatively small percentage of the total residue concentration (Table 5), ranging from 10 to 17% (measured values) or from 16 to 25% (with correction for recoveries). Data on residues at 28 days postapplication, which were collected to cover the possible need for a longer PHI, show residues up to a maximum of 3.39 mg/kg (Victoria, 1997).

Effect of Washing on Residue Concentration. Although it is stated in Tomlin (1994) that chlozolinate is a systemic pesticide, the data given in Table 3 show that considerable amounts of the parent compound,

Table 3. Residues (Milligrams per Kilogram) of Chlozolinate on Grapes before and after Washing

variety	year	days after treatment	unwashed grapes		washed grapes		% loss on washing	mean % loss ± SD
			residues[a]	RSD	residues[a]	RSD		
Cardinal	1996[b]	0	4.29	31	1.85	24	57	
		4	2.06	69	0.96	53	53	
		8	2.40	47	1.13	109	53	
		14	3.31	73	0.90	42	73	
		21	1.22	22	0.25	91	80	
		28	0.54	36	0.13	73	76	65 ± 12
	1997	0	11.3	9.1	4.5	16	60	
		4	10.0	3.6	4.6	10	54	
		8	9.22	14	3.1	19	66	
		14	5.62	4.9	2.4	8.5	57	
		21	3.41	18	1.4	13	59	
		28	1.63	9.2	0.57	10	65	60 ± 5
Victoria	1996	0	5.07	17	1.09	19	79	
		4	4.49	18	1.25	21	72	
		8	3.54	35	1.11	55	69	
		15	1.57	38	0.66	78	58	
		21	1.54	27	0.39	13	75	
		28	1.42	45				71 ± 8
	1997	0	11.1	15	6.11	6.3	45	
		4	9.18	10	5.36	17	42	
		8	8.88	8.4	3.76	17	58	
		14	4.60	4.3	2.43	10	47	
		21	4.23	10	2.35	13	44	
		28	3.39	16	1.19	24	65	50 ± 9

[a] Mean of three samples, each analyzed in duplicate. [b] Residues adjusted for the effect of growth.

Table 4. Residues (Milligrams per Kilogram) of Metabolite S1 on Grapes before and after Washing

variety	year	days after treatment	unwashed grapes residues[a]	unwashed grapes RSD	washed grapes residues[a]	washed grapes RSD	% loss on washing	mean % loss ± SD (days 4−28)
Cardinal	1996	0	0.30	28	0.17	31	43	
		4	0.39	45	0.47	32	−21[b]	
		8	0.62	5.6	0.40	34	35	
		14	0.49	48	0.40	46	18	
		21	0.25	31	0.19	63	24	
		28	0.07	51	0.08	41	−14[b]	9 ± 25
	1997	0	0.85	9.7	0.37	28	56	
		4	0.81	9.8	0.85	12	−5[b]	
		8	1.07	15	0.95	17	11	
		14	0.93	9.1	0.92	5.3	1	
		21	0.64	14	0.64	3.9	0	
		28	0.28	6.5	0.24	23	14	4 ± 8
Victoria	1996	0	0.56	15	0.33	6	41	
		4	0.58	23	0.63	20	−9[b]	
		8	0.79	25	0.54	31	32	
		15	0.27	37	0.35	81	−30[b]	
		21	0.17	21	0.11	19	35	
		28	0.14	35				7 ± 32
	1997	0	1.10	10	0.81	24	26	
		4	1.16	10	1.02	3.2	12	
		8	1.02	20	0.84	15	18	
		14	0.81	10	0.67	8.8	17	
		21	0.53	3	0.52	15	2	
		28	0.56	11	0.37	6.9	34	12 ± 17

[a] Mean of three samples, each analyzed in duplicate. [b] Increase in residue concentration probably due to sampling variability in deposits.

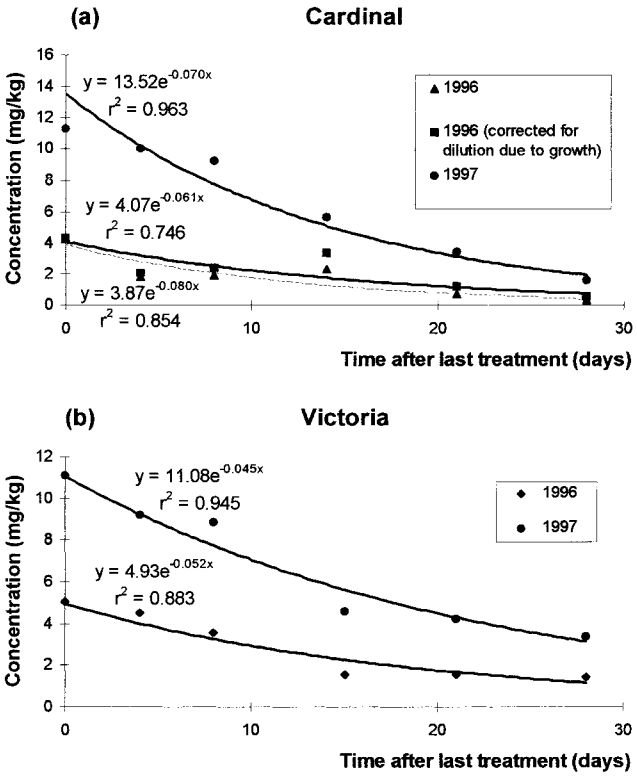

Figure 3. Dissipation of chlozolinate in 1996 and 1997 on (a) Cardinal grapes and (b) Victoria grapes.

between 42 and 80%, are removed by washing. The reduction in residues shows no trend with time, and no differences were observed between the two varieties of grapes. The average loss of chlozolinate due to washing for each variety and year is also given in Table 3. In contrast, the data for S1 given in Table 4 indicate that the metabolite rapidly becomes incorporated into the plant tissues, with significant amounts of residue being removed by washing only on day 0. Despite rather large

Table 5. Percentage Contribution[a] of Metabolite S1 to the Total Residue (Chlozolinate + S1)

day	Cardinal 1996	Cardinal 1997	Victoria 1996	Victoria 1997
0	7	7	10	9
4	16	8	11	11
8	21	10	18	10
14	13	14	15	16
21	17	16	10	11
28	11	15	9	14

[a] Measured values.

variation in the data, the averages of the amounts of S1 removed from day 4 to day 28 (Table 4) for each year and variety are close to 0, indicating that significant amounts of S1 do not remain on the surface of the grapes. These results are consistent with those obtained by the manufacturer, ISAGRO, after application of radiolabeled chlozolinate to grapes of variety Labrusco (^{14}C-chlozolinate labeled in two carbonyl groups, one of which belonged to the ester group and the other to the carbonyl group in the 4-position of the oxazolidinic ring). According to these data, most of the total residue on the surface of the berries consisted of the parent compound and could be readily removed by washing, leading to the conclusion that chlozolinate has a limited capacity of penetration into the tissues. Traces of metabolite S1 were also found as early as 1 day after treatment (Santi et al., 1983). However, a similar study on strawberries gave contradictory results, suggesting quick penetration of the radioactive molecule into the fruits (Santi et al., 1981). It thus becomes apparent that, from the residue behavior point of view, the systemicity or not of a plant protection product (degree, extent, and rate of penetration and translocation) should be evaluated on the basis of specific studies on a wide variety of crops. The present data on grapes, together with those of Santi et al. (1983), lead to the conclusion that the actual intake of chlozolinate from grapes by consumers

is likely to be considerably less than the theoretical, given the significant reduction in residues achieved by washing.

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